

Chromosome 2 (2p16) abnormalities in Carney complex tumours

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Carney complex (CNC) is an autosomal dominant multiple endocrine neoplasia and lentiginosis syndrome characterised by spotty skin pigmentation, cardiac, skin, and breast myxomas, and a variety of endocrine and other tumours. The disease is genetically heterogeneous; two loci have been mapped to chromosomes 17q22-24 (the *CNC1* locus) and 2p16 (*CNC2*). Mutations in the *PRKARIA* tumour suppressor gene were recently found in *CNC1* mapping kindreds, while the *CNC2* and perhaps other genes remain unidentified. Analysis of tumour chromosome rearrangements is a useful tool for uncovering genes with a role in tumorigenesis and/or tumour progression. CGH analysis showed a low level 2p amplification recurrently in four of eight CNC tumours; one tumour showed specific amplification of the 2p16-p23 region only. To define more precisely the 2p amplicon in these and other tumours, we completed the genomic mapping of the *CNC2* region, and analysed 46 tumour samples from CNC patients with and without *PRKARIA* mutations by fluorescence in situ hybridisation (FISH) using bacterial artificial chromosomes (BACs). Consistent cytogenetic changes of the region were detected in 40 (87%) of the samples analysed. Twenty-four samples (60%) showed amplification of the region represented as homogeneously stained regions (HSRs). The size of the amplicon varied from case to case, and frequently from cell to cell in the same tumour. Three tumours (8%) showed both amplification and deletion of the region in their cells. Thirteen tumours (32%) showed deletions only. These molecular cytogenetic changes included the region that is covered by BACs 400-P-14 and 514-O-11 and, in the genetic map, corresponds to an area flanked by polymorphic markers D2S2251 and D2S2292; other BACs on the centromeric and telomeric end of this region were included in varying degrees. We conclude that cytogenetic changes of the 2p16 chromosomal region that harbours the *CNC2* locus are frequently observed in tumours from CNC patients, including those with germline, inactivating *PRKARIA* mutations. These changes are mostly amplifications of the 2p16 region, that overlap with a previously identified amplicon in sporadic thyroid cancer, and an area often deleted in sporadic adrenal tumours. Both thyroid and adrenal tumours constitute part of CNC indicating that the responsible gene(s) in this area may indeed be involved in both inherited and sporadic endocrine tumour pathogenesis and/or progression.

Carney complex (CNC, OMIM 160980) is an autosomal dominant syndrome characterised by multiple neoplasias, including cardiac, skin, and breast myxomas, endocrine and other tumours, and spotty skin pigmentation in the form of lentigines, common and blue naevi, and other lesions.^{1,2} The disease is genetically heterogeneous and has been mapped to 2p16 (*CNC2*) and 17q22-24 (*CNC1*).^{3,4} The *CNC1* gene was recently identified as the tumour suppressor gene *PRKARIA*, coding for the type 1A regulatory subunit of the protein kinase A (PKA) holoenzyme, a critical component of numerous cellular signalling systems; inactivating mutations of this gene have been found in approximately half of the CNC kindreds studied to date.^{5,6}

The *CNC2* locus was mapped to the 2p16 region by linkage analysis; the originally identified region spanned approximately 4 cM of genetic distance and a maximum two point lod score was found for the *CA2/D2S123* locus.³ Additional genetic mapping narrowed this region to an approximately 2 Mb long area between markers *D2S2153* and *D2S1352*.⁷ To characterise this region and isolate candidate genes, the 2p15-21 area was cloned and a yeast artificial chromosome (YAC)-bacterial artificial chromosome (BAC) map covering approximately 10 Mb of physical distance between markers *D2S378* and *D2S391* on the centromeric and the telomeric sides, respectively, was generated.⁸ A number of candidate expressed sequences from the region have been identified and excluded^{6,9-11} (Kirschner and Stratakis, unpublished data). Following the identification

of the gene responsible for CNC in 17q mapping families,³ all of the available CNC kindreds, including those linked to 2p16,³ were screened for *PRKARIA* mutations. Phenotyping errors were identified in only one family (CAR108); a second family (CAR01) that did not map to 2p16 but was included in the original cohort³ was one of the families that was used to identify the *CNC1* locus; affected members in this family were all found to have the relatively frequent 578delTG *PRKARIA* mutation.⁵ Thus, of 11 kindreds that had been mapped collectively to 2p16,³ nine had no *PRKARIA* defects; linkage to 2p16 was reaffirmed with newer markers for the *CNC2* region for at least six of these kindreds.⁶

The association of specific chromosomal abnormalities with particular human cancers has been shown to be valuable in identifying genes involved in tumorigenesis and tumour progression.^{12,13} For example, regions of chromosomal amplification may contain oncogenes; deleted regions may be associated with loss or inactivation of tumour suppressor genes.¹⁴ Gene amplification is a frequent abnormality observed in a wide range of solid tumours and may involve several loci and/or genes, including multiple amplicons in breast cancer,¹⁵⁻¹⁹ the 12p and 11q13 amplification in osteosarcomas,²⁰ and oral and other head and neck squamous cell carcinomas (OSCC and HNSCC),^{21,22} respectively, to cite just a few examples. Oncogenes may be present in these amplicons, the most characteristic example being the *MYCN* gene in neuroblastomas.²³

Table 1 CGH abnormalities in CNC tumours

Case	Kindred	Tumour pathological diagnosis	Locus	Mutation of <i>PRKARIA</i>	CGH results	
					Gains	Deletions
1	CAR01	Ovarian cyst	<i>CNC1</i>	c.578delGT	2p; 2q36; 9q34; 19p; 19q	5p; 6
2	CAR01	Pituitary adenoma	<i>CNC1</i>	c.578delGT	1p; 2p; 4qter; 8p; 17; 16p-qter; 12q; 19; 20; 22	6q; 7q; 11
3	CAR03	PPNAD	<i>CNC2</i>	–	20p	6q22-qter; 10p; 9qter
4	CAR110	Facial myxoma	<i>CNC1</i>	c.769C>T	2p16-23; 5q (middle)	11
5	CAR110	Thyroid carcinoma	<i>CNC1</i>	c.769C>T	2p	
6	CAR503.01	PPNAD	Sporadic	Exon 2IVS-2A>G	1pter-p (middle); 16qter-q (middle); 19; 20q; 22	4cen-qter
7	CAR515.01	PPNAD	Sporadic	–	9q34	
8	CAR06.03	Skin myxoma	Sporadic	–	2p	

The first column of the table provides the designated CAR code for each family assigned by the investigators. The second column shows the histological diagnosis of the tumour studied. The third column lists the genetic locus (*CNC1* versus *CNC2*). "Sporadic" indicates a case with no known family history and presumably de novo mutation of the gene responsible for CNC in this subject. The fourth column lists the results of *PRKARIA* mutation analysis. The fifth column presents the CGH results. PPNAD=primary pigmented nodular adrenocortical disease.

Gene amplification may be detected at the cytogenetic level either extrachromosomally, as double minute chromosomes (DMs) (small, dot-like chromosome masses that can contain thousands of copies of an oncogene²⁴) or intrachromosomally as homogeneously staining regions (HSRs) that are frequently integrated in abnormal chromosomes.²⁵

In the present study, we first examined eight CNC tumours by CGH, a technique that allows the detection of large changes in tumour DNA (table 1).²⁶ We detected a low level 2p amplification as a recurrent event in four of eight samples; in one tumour the region amplified was specifically 2p16-p23. To define more precisely the 2p amplicon in these and other tumours, we extended the genomic mapping of the 2p15-21 region, identified new BACs that map to this locus, and used them to perform fluorescence in situ hybridisation (FISH) on samples from tumours from patients with CNC. Frequent changes of the 2p16 region were identified, interestingly in patients with germline *PRKARIA* mutations, too. Most tumours showed amplification of the area, in the form of HSRs, suggesting that an oncogene may lie at this chromosomal location.

MATERIALS AND METHODS

Subjects and tissues

We studied 46 tumours from familial (34) and sporadic (12) cases of CNC. The patients and the histology of their tumours are listed in table 2. All subjects gave informed consent for analysis of their tissue and/or DNA under protocol 95-CH-0059 of the National Institute of Child Health and Human Development. Specimens were obtained following biopsy and/or surgical resection and were snap-frozen at -70°C or used immediately for "touch preparations". Short term cultures were established from several lesions. For three tumours (cases 5, 18, and 31), touch preparations and cultures were available. Because of the limited amount of tissue obtained, in 12 cases only cultures were available for FISH analysis. When available, samples of surrounding normal tissue were taken for use as controls in each experiment. As additional FISH controls we also used prostatic adenoma tumours (n=4) obtained from patients with no known history of any familial disease, as well as pancreatic (CRL-1420) and breast (HTB-22 and HTB-30) cancer cell lines received from The American Type Culture Collection (Manassas, VA).

Touch preparations of the tumours were fixed in methanol-acetic acid (3:1) for 20 minutes, air dried, and equilibrated in $2 \times \text{SSC}$ (0.3 mmol/l NaCl, 30 mmol/l sodium citrate (pH 7.0)) solution, followed by dehydration in ethanol series of 70, 80, 90, and 100%. Metaphase chromosomes were prepared from tumour cell lines using standard harvesting procedures.

Genomic comparative hybridisation (CGH)

CGH was performed as described previously.^{26, 27} Genomic DNA was extracted from the frozen tissues using the Qiagen kit

(Qiagen Inc, Chatsworth, CA). Human reference DNA was extracted from peripheral blood lymphocytes of a normal female donor. Tumour and reference DNA was labelled by biotin-11-dUTP and digoxigenin-11-dUTP (Roche Molecular Biochemicals, Mannheim, Germany), respectively. One μg of each of the labelled probes was hybridised in the presence of excess Cot-1 and herring sperm DNA (Life Technologies, Gaithersburg, MD) to metaphase chromosomes prepared from a healthy subject. The biotin labelled tumour genome was visualised with avidin conjugated to fluorescein isothiocyanate (Vector Laboratories Inc, Burlingame, CA), and the digoxigenin labelled reference DNA was detected with antidigoxigenin rhodamine (Roche Molecular Biochemicals, Mannheim, Germany). Chromosomes were counterstained with DAPI (4', 6'-diamidino-2-phenylindol-dihydrochloride). Digital acquisition of grey scale images of the fluorescein isothiocyanate labelled tumour DNA, the tetra-methylrhodamine isothiocyanate labelled reference DNA, and the DAPI counterstain was done using a cooled charge coupled device camera connected to a Leica microscope equipped with fluorochrome specific optical filters. IP Lab Image software (Scan Analytics Corp, VA) was used for image acquisition. The profiles of the tumour:reference fluorescence intensity ratios were generated using Vysis Quantitative Image Processing System (QUIPS CGH). Average ratio profiles were computed as the mean value of at least eight ratio images to identify chromosomal copy number changes in all cases.^{28, 29}

Genomic mapping of bacterial artificial chromosomes (BACs) on 2p16

BACs that have been characterised and mapped to the chromosome 2p15-21 region,⁸ as well as newly identified clones were used in the experiments. Fig 1 displays these and their location on the 2p16 genomic map, based on the 2p15-21 map that was described elsewhere.⁸ The current map consists of over 100 large insert clones and contains over 150 polymorphic and non-polymorphic STS. It covers approximately 10 Mb of physical distance between the markers D2S378 at the centromeric end and D2S391 at the telomeric end. In the process of the work, 41 new STSs were generated and placed on the map²⁶ (Kirschner and Stratakis, unpublished data).

Briefly, the isolation of new BACs and STS/EST content mapping was done as follows: polymerase chain reaction (PCR) analyses of all DNA samples were carried out under standardised conditions consisting of 10 μl reactions that contained $1 \times \text{PCR}$ buffer I (Perkin-Elmer, Foster City, CA), 200 $\mu\text{mol/l}$ each dNTP, 0.6 $\mu\text{mol/l}$ of forward and reverse primers, 50 ng DNA, and 0.5 U *Taq* polymerase. The reaction mixtures also contained 12% sucrose and 180 nmol/l cresol red to facilitate direct loading of the samples after PCR. Cycling reactions were performed with an initial five minute denaturation step at 95°C , followed by 30 cycles of 95°C for one minute, 56°C for one minute, and 72°C for one minute. Primer sets that did not

Table 2

Case	Kindred	Tumour pathological diagnosis	Locus	Mutation of <i>PRKARIA</i>	2p16 results
1	CAR01	Ovarian cystic adenoma	<i>CNC1</i>	c.578delGT	Ampl
2	CAR01	Uterine fibromyxoma	<i>CNC1</i>	c.578delGT	Ampl
3	CAR01	Myxoid lipoma	<i>CNC1</i>	c.578delGT	Ampl
4	CAR01	PPNAD	<i>CNC1</i>	c.578delGT	Ampl
5	CAR01	Thyroid papillary carcinoma	<i>CNC1</i>	c.578delGT	Ampl
6	CAR01	PPNAD	<i>CNC1</i>	c.578delGT	Ampl
7	CAR01	Pituitary adenoma	<i>CNC1</i>	c.578delGT	Ampl
8	CAR01	Skin myxoma	<i>CNC1</i>	c.578delGT	Amp/Del
9	CAR02	PPNAD	Sporadic	NA	Del
10	CAR03	PPNAD	<i>CNC2</i>	-	Del
11	CAR06	Heart myxoma	Sporadic	-	Ampl
12	CAR06	Skin myxoma (CL)	Sporadic	-	Del
13	CAR07	PPNAD	Sporadic	-	Ampl
14	CAR07.04	PPNAD	Sporadic	-	Del
15	CAR14	PPNAD	Sporadic	-	Amp/Del
16	CAR14	PPNAD	Sporadic	-	Del
17	CAR16	Pituitary adenoma (CL)	NA	-	Ampl
18	CAR16	Thyroid follicular adenoma (CL)	NA	-	Ampl
19	CAR19	PPNAD	NA	c.88A>G	Ampl
20	CAR20	Pituitary adenoma	<i>CNC1</i>	c.578delGT	Ampl
21	CAR20	Schwannoma (CL)	<i>CNC1</i>	c.578delGT	NI
22	CAR20	Skin myxoma (CL)	<i>CNC1</i>	c.578delGT	NI
23	CAR25.03	PPNAD	<i>CNC1</i>	Exon8IVS+3G>A	Del
24	CAR25.04	PPNAD (CL)	<i>CNC1</i>	Exon8IVS+3G>A	NI
25	CAR36.03	PPNAD	Sporadic	-	Del
26	CAR43.03	PPNAD	Sporadic	NA	Amp/Del
27	CAR 45	Skin myxoma	Sporadic	-	Del
28	CAR102	Heart myxoma	NA	-	Ampl
29	CAR110	Ovarian cyst	<i>CNC1</i>	c.769C>T	Del
30	CAR110	Ovarian cyst (CL)	<i>CNC1</i>	c.769C>T	Ampl
31	CAR110	PPNAD	<i>CNC1</i>	c.769C>T	Ampl
32	CAR110	Heart myxoma	<i>CNC1</i>	c.769C>T	Ampl
33	CAR110	Pituitary adenoma	<i>CNC1</i>	c.769C>T	Ampl
34	CAR110	Cheek myxoma	<i>CNC1</i>	c.769C>T	Ampl
35	CAR110	LCCSCT (CL)	<i>CNC1</i>	c.769C>T	NI
36	CAR110	Thyroid follicular carcinoma	<i>CNC1</i>	c.769C>T	Del
37	CAR110	Skin myxoma	<i>CNC1</i>	c.769C>T	NI
38	CAR503.01	PPNAD	Sporadic	Exon2IVS-2A>G	Ampl
39	CAR506.01	PPNAD	Sporadic	c.211C>T	Del
40	CAR506.01	Heart myxoma (CL)	Sporadic	c.211C>T	NI
41	CAR508.01	Ovarian carcinoma	Sporadic	-	Del
42	CAR509.01	PPNAD	Sporadic	-	Ampl
43	CAR512.01	PPNAD	Sporadic	-	Ampl
44	CAR513.01	Gluteal myxoma	Sporadic	Intron3IVS+1G>C	Ampl
45	CAR515.01	PPNAD	Sporadic	-	Ampl
46	CAR521.01	PPNAD	Sporadic	-	Del

The first column of the table gives the assigned CAR code. The second column provides the histological diagnosis of the tumour studied; if a cell line was studied this is indicated in parentheses (CL). The third column provides the genetic locus information (*CNC1* versus *CNC2*). "Sporadic" indicates a case with no known family history, as above. The fourth column shows the results of *PRKARIA* mutation analysis. The fifth column presents the results of the study. Ampl=amplification, Del=deletion, NA=not analysed, NI=normal (two signals of BAC analysed), LCCSCT=large cell calcifying Sertoli cell tumour, PPNAD=primary pigmented nodular adrenocortical disease.

generate reproducible bands from whole human genomic DNA were not considered for further use. For EST/STS content mapping, BAC DNAs were prepared from 5 ml overnight cultures, using commercially available kits (Puregene, Gentra Biosystems, Minneapolis, MN) and spin miniprep columns (Qiagen Corp, Valencia, CA), respectively.

To obtain BAC end sequences, DNA samples from individual clones were purified using a modified alkaline lysis procedure.³⁰ DNA purified in this way was sequenced directly using the T7 primer or an SP6 end primer (AAGGGTTTGGG) with Big-Dye Terminators (Perkin-Elmer, Foster City, CA). BAC end sequences were then submitted to the Whitehead Institute's Primer3 software (http://www.genome.wi.mit.edu/genome_software/other/primer3.html) to select 20 base pair (bp) PCR primers that were not complementary to known repetitive sequences.

Pulsed field gel electrophoresis was used to analyse the size of BAC inserts. BAC DNA was prepared for sequencing as described above and digested in solution with *NotI* before electrophoresis. All pulsed field analyses were performed on a

CHEF Mapper II apparatus (Biorad Laboratories, Hercules, CA), using programs recommended by the manufacturer to resolve DNA fragments of the appropriate size.

Fluorescence in situ hybridisation (FISH)

FISH was performed as described elsewhere.³¹ In brief, DNA was labelled by digoxigenin-11-dUTP or biotin-11-dUTP by nick translation followed by anti-dig-rhodamine or avidin-FITC detection. After precipitation in ethanol in the presence of 50 × herring sperm DNA and 50 × Cot-1 fraction of human DNA, the DNA pellet was resuspended in Hybridzol solution (50% deionised formamide/10% dextran sulphate/2 × SSC) with a final concentration of 25 mg/ml. For dual colour FISH, the concentration of each probe was 50 mg/ml. After the detection reaction, cells were counterstained with DAPI. Hybridisation signals were analysed using a Leica epifluorescence microscope; fluorescence images were automatically captured on a Photometrics cooled CCD camera (Photometrics Ltd, Tuscon, AZ), using IP Lab Image software (Scanalytics Inc, Fairfax, VA). At least 100 non-overlapping cells with

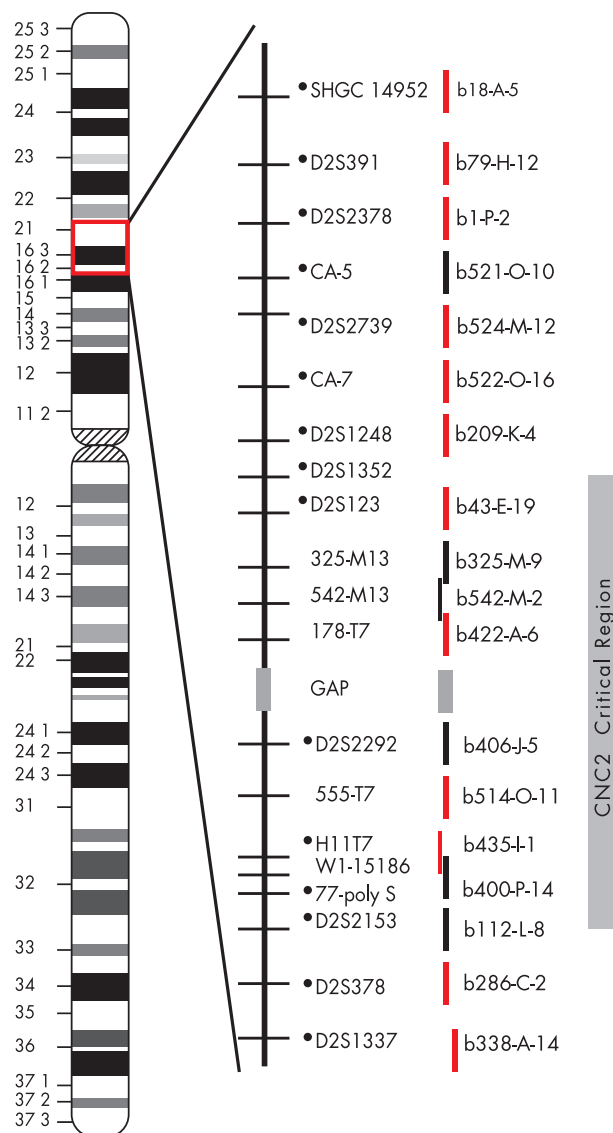


Figure 1 Ideogram of chromosome 2 (left) with microsatellite markers (middle vertical line) and BAC clones (right). Polymorphic markers are marked by asterisks; previously undescribed BAC clones are indicated in red. All STSs and remaining BACs have been described by Kirschner *et al.*⁸

strong hybridisation signals were scored per case. The sensitivity of the probes was assessed by FISH to the karyotypically normal human dermal fibroblast cell line CC-2511 (Clonetics).

For all BACs chosen for analysis, FISH results were very similar with insignificant variations. For example, for BAC400-P-14, 90% of nuclei showed two clear signals, 1% showed no signal, 3% showed one signal, 3% three signals, and 3% four signals. In the analysis of tumour cells, the 2p16 region was classified as amplified if more than 5% of cells contained multiple signals of the probe being tested and two copies of chromosome 2. The presence of more than 20% cells with only one BAC signal was interpreted as an allelic deletion. Normal tissues from patients showed less than 8% of cells with one BAC signal varying insignificantly between samples. Control probes from other chromosomes (see below) were also hybridised to tumour cells and showed two expected signals in more than 90% of the cells.

For all FISH experiments, control probes from chromosomes 1, 4, 10, 16, and 22 were used. All BACs were obtained from a commercially available library (Research Genetics).

DNA was extracted as described elsewhere.³⁰ A chromosome 2 specific centromeric α -satellite probe (Vysis) or a painting probe specific for chromosome 2 were also used for chromosome identification.

Statistical analysis

For all analyses, χ^2 test with Fisher's correction, when appropriate, was performed using the Statistica software (StatSoft, Tulsa, OK).

RESULTS

CGH

We performed CGH on eight samples of tumours from CNC patients, as a first screening technique to detect possible large scale chromosomal aberrations associated with these lesions. These results are presented in table 1.

The most frequent changes were gains, whereas losses were detected in only five of eight samples. The greatest number of changes were detected in cases 1, 2, and 6 (ovarian cyst, pituitary adenoma, and PPNAD). Three samples (cases 5, 7, and 8) showed amplification only of a single region. Changes detected in more than one tumour involved losses of 6q and 11, whereas chromosomes 2, 9, 16, 19, 20, and 22 were involved in repeatedly identified gains. In total, 13 chromosomal regions showed amplification, but in six the amplification was seen in only one sample. The most frequent and greatest contiguous change was amplification of 2p, which was detected in four of eight samples. One of the samples had a relatively narrow region of amplification that was defined as containing the 2p16-p23 region.

Identification of new BACs

A total of 12 new BACs, identified by STS to map to the previously described 2p15-21 contig,⁸ are presented in fig 2. They complement the existing map, but leave unclosed the known central gap. Their location was confirmed by fiber-FISH (data not shown) and was correlated with known BACs.

Amplification of the 2p16-21 region

In order to define more precisely the region amplified on 2p, we used FISH with BACs from the 2p15-p21 region. Table 2 presents results of FISH on 46 tumour samples. Abnormalities were detected in the 34 tumours studied but only in six of the 12 cell lines; the difference was statistically significant ($p < 0.05$). FISH detected amplification of the region in 24 (60%) of 40 tumours that showed abnormalities (fig 3C-F). Three tumours (8%) showed both amplification and deletion of the region in their cells. To define the extent of the region of amplification, we used dual colour FISH with BAC probes from the centromeric and telomeric borders of 2p16-21. BACs 338-A-14 and 18-A-5 were not amplified, establishing the centromeric and telomeric boundaries of the amplified region in the tumours above D2S1337 (2p16.1) and below SHGC14952 (2p21), respectively (fig 3B). The borders of this amplicon were flanked by probes b1-p-2 (D2S2378) and b286-c-2 (D2S378), although the sizes of amplicons varied between tumours and between cells in one tumour. For example, an amplicon in cases 15 and 7 was flanked by the BAC probes mentioned above, from the centromeric and telomeric borders of the 2p15-21 region, respectively. This region of amplification included therefore more than 10 Mb of genomic distance, far above and below the CNC2 critical region. In case 32, on the other hand, the amplicon included only BAC 400-P-14 and its most proximal BAC514-O-11; characteristically, however, BAC 514-O-11 was amplified in a much smaller fraction of the cells than BAC 400-P-14.

The level of amplification also varied from sample to sample and frequently within the same tumour (fig 3F). Also, some cells displayed an amplification of one or both alleles. A high level of amplification was detected in only three tumours

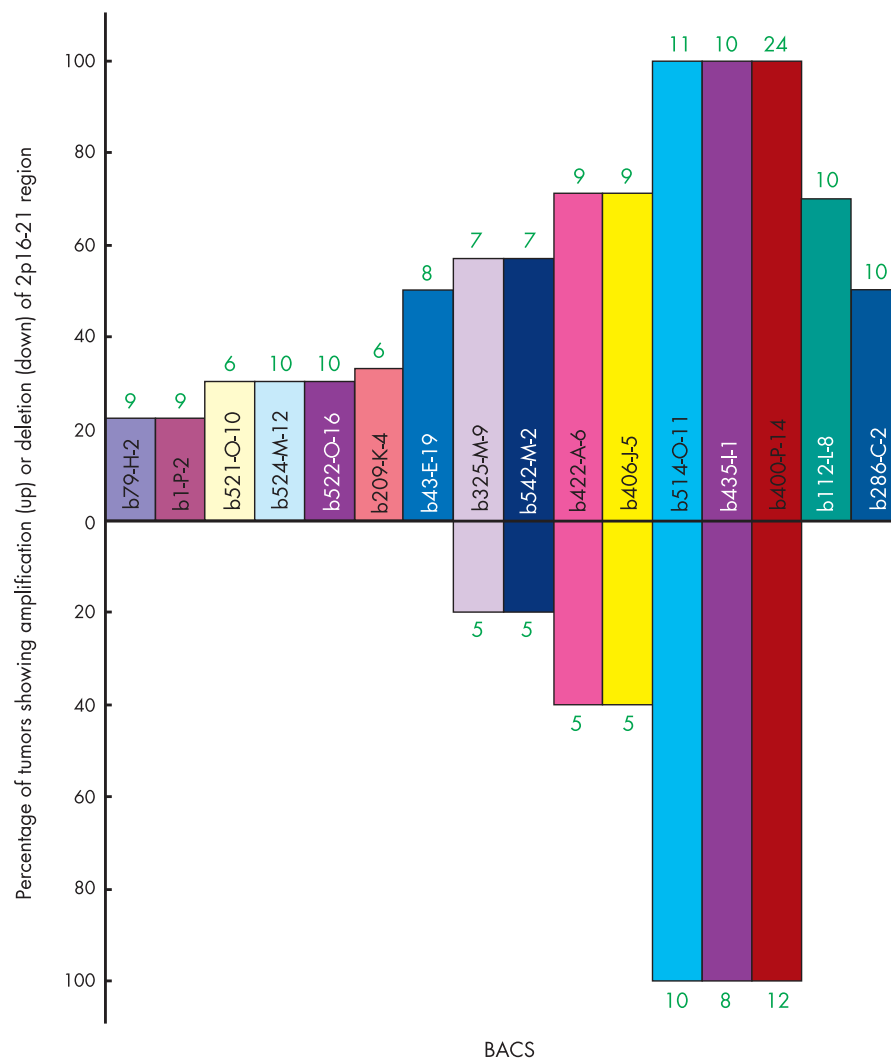


Figure 2 FISH analysis of CNC tumours with BACs from the 2p16-21 region. The top and the bottom halves of the figure represent the percentage of the samples that showed amplification (up) or deletion (down) of the region. The name of each BAC clone is shown across the columns and the number of tumours analysed is shown at the top or the bottom of the column. The area of most consistent changes is defined by BAC 400-P-14, 435-I-1, and 514-O-11 (D2S2251-D2S2292) suggesting that it contains a putative candidate(s) involved in *CNC2*.

(cases 2, 17, and 33); the majority of the specimens showed low or mid-level amplification counting 4-20 copies of the probe.

Probes displayed a tandem pattern of amplification in the interphase nuclei indicating the presence of HSRs, which were seen as a side by side duplication of dual colour BAC signals (fig 3C, D). Metaphase FISH analysis of a cell line established from a cheek myxoma (case 34) showed cells with differential signal intensity of the 2p16 region (fig 3E).

The region of most consistent amplification was defined by BAC clones 400-P-14, 435-I-1, and 514-O-11 (D2S2251-D2S2292). These BACs were included in the amplicon of all tumours that displayed amplification. They also had a greater copy number than the BACs used in the present study (fig 2); in one tumour, BACs 400-P-14 and 435-I-1 showed higher level of amplification and in a greater number of cells than BAC 514-O-11, although in all other tumours these three BACs had a similar degree of amplification.

Control experiments for the amplification

FISH analysis with α -satellite probe specific for chromosome 2 centromere detected two copies of the chromosome in all tumours analysed indicating that the cells were diploid. In addition, the 2p16 BACs that showed copy number gain on CNC tumours were hybridised to normal tissues from the

same patients; the expected two copies were found in all cells. BAC clones from chromosomes 1, 10, 16, and 22 when hybridised to CNC tumours also showed the expected two signals. Additionally, control experiments included hybridisation of BACs 400-P-14 and 514-O-11 to prostate adenoma tumour cells (fig 4D), breast cancer cell lines HTB22 and HTB30, and pancreatic cancer cell line CRL1240. Two signals of equal intensity were shown in more than 90% of prostate adenoma cells examined. Breast cancer cell lines HTB22 and HTB30 showed equal copy numbers of the BACs and α -satellite probe specific for chromosome 2 centromeres. Pancreatic cancer cells also showed no amplification of BACs 400-P-14 and 514-O-11, although we detected a translocation of the region (data not shown). Taken together, the data allowed us to conclude that the cytogenetic changes of the 2p16 region were specific to CNC.

Deletion of 2p16 region

In addition to amplification of the 2p16-21 region in CNC tumours, we also detected an allelic deletion of the region in 13 tumours (32%); amplification and deletion were seen in three tumours (table 2). Some tumours, primarily those of adrenocortical origin, lost the whole chromosome 2 as shown by FISH experiments with a chromosome 2 specific centromeric α -satellite probe or a chromosome 2 specific painting

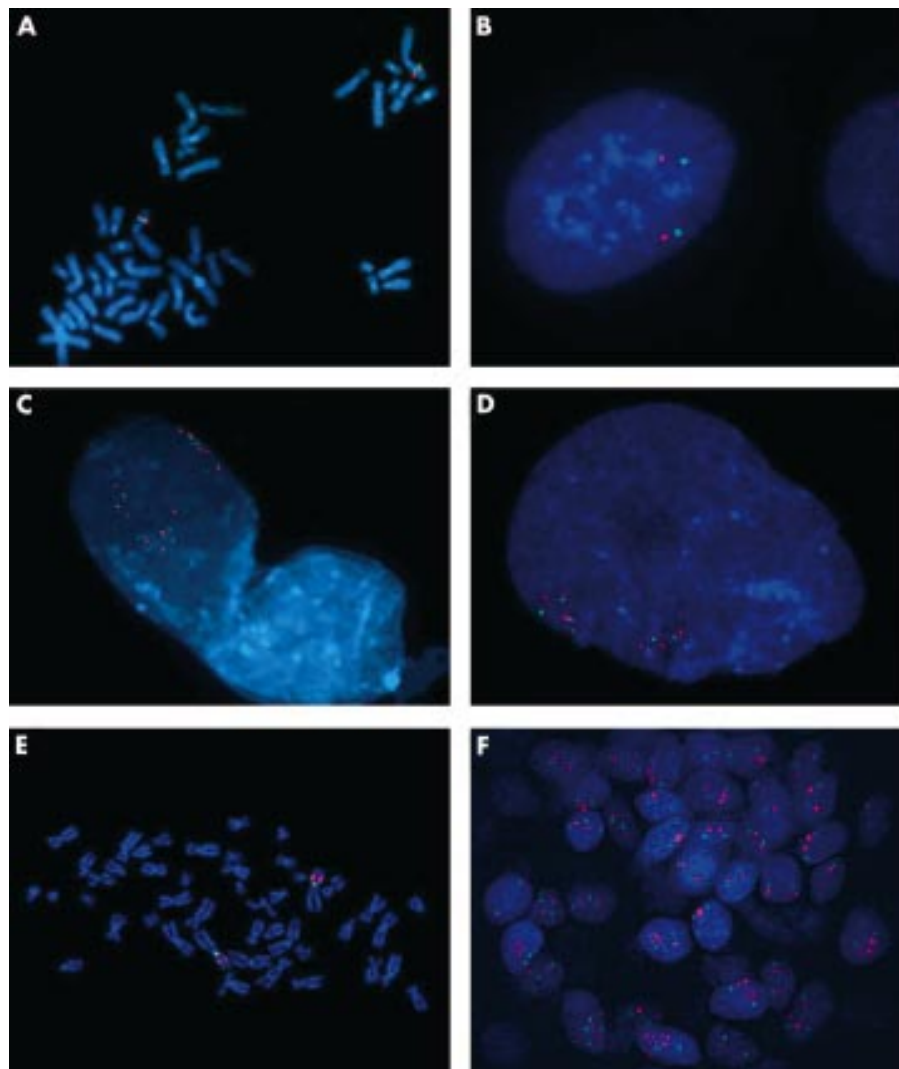


Figure 3 Amplification of the 2p16-21 region in CNC tumours. (A) Mapping of the two clones used in this study to chromosome 2 (2p16-21) by FISH on a metaphase spread; the green and red signals correspond to BAC 209-K-4 (D2S1248) and BAC 1-P-2 (D2S2378). (B) An ovarian tumour cell (case 1) hybridised with BACs 18-A-5 (red) and 338-A-14 (green) containing SHGC14952 (2p21) and D2S1337 (2p16.1). These BACs were not amplified in CNC tumours establishing the boundaries of amplified region. (C) A cell from the same tumour as in (B) after hybridisation with BACs 43-E-9 (red) and 79-H-2 9 (green) showed multiple signals of each colour. (D) FISH of BACs 112-I-8 and 522-O-16 hybridised to a pituitary adenoma cell (case 17) detected amplification of both BACs. (E) BAC 400-P-14 hybridised to metaphase cells of a short term culture of a cheek myxoma (case 34). A consistent differential signal intensity of chromosome 2 supports amplification of the region 2p16. (F) Gluteal myxoma cells (case 44) hybridised with BAC 400-P-14 (red) and α -satellite probe specific for chromosome 2 (green). Cells showed different levels of amplification of BAC400-P-14 (red); in these cells, the centromeric α -satellite probe showed two signals. Some cells were normal, showing two copies of both probes; other cells showed three or four copies of both probes, representing triploid and tetraploid (or dividing) cells.

probe. However, most of the samples showed two copies of chromosome 2 in their cells.

The size of the deleted region in these tumours varied, but it was generally considerably smaller than the region of amplification. It was flanked by BACs 325-M-13 (2p16.3) and 400-P-14 (2p16.1) from the telomeric and centromeric sides, respectively (fig 1). Dual colour FISH with BACs from within the region narrowed the boundaries of the deleted region. For example, FISH of BACs 400-P-14 and 422-A-6 (fig 4B) or 400-P-14 and 542-M-2 to PPNADs from cases 10 and 36, respectively, showed a deletion only of BAC 400-P-14, whereas BACs 422-A-6 and 542-M-2 were present in two copies. FISH with BACs 400-P-14 and 406-J-5 showed a deletion of BAC 400-P-14, but BAC406-J-5 showed two signals in cases 10 and 29 (fig 4F). FISH of BACs 400-P-14 and 514-O-11 (fig 4C) to skin myxoma cells (case 12) showed a deletion of both BACs establishing the minimal size of the deleted region in CNC tumours (fig 2). BAC 435-I-1 that was the nearest one to BAC

400-P-14 (and slightly overlaps with it) on the physical map of the 2p16 region was lost in CNC tumours together with BAC 400-P-14. BAC 112-I-8 that maps more centromeric to BAC-400-P-14 showed two signals in all tumours analysed.

Taken together, these data suggested that the region covered by BACs 400-P-14, 435-I-1, and BAC 514-O-11 most probably contained the *CNC2* locus. Based on the sizes of BACs 400-P-14, 435-I-1 and 514-O-11 (160, 125, and 160 kb, respectively) and taking into account the overlapping of BACs 400-P-14 and 435-I-1 on the published map,⁶ we can assume that the size of this region is approximately 400-450 kb.

Control experiments for the deletions

Prostate adenoma tumours used as controls showed less than 10% of cells with one signal of BACs 400-P-14 or 514-O-11. FISH with other 2p16 BACs also showed the expected two signals. Breast cancer cell lines HTB22 and HTB30 showed equal copy numbers of these BACs and α -satellite probes that are

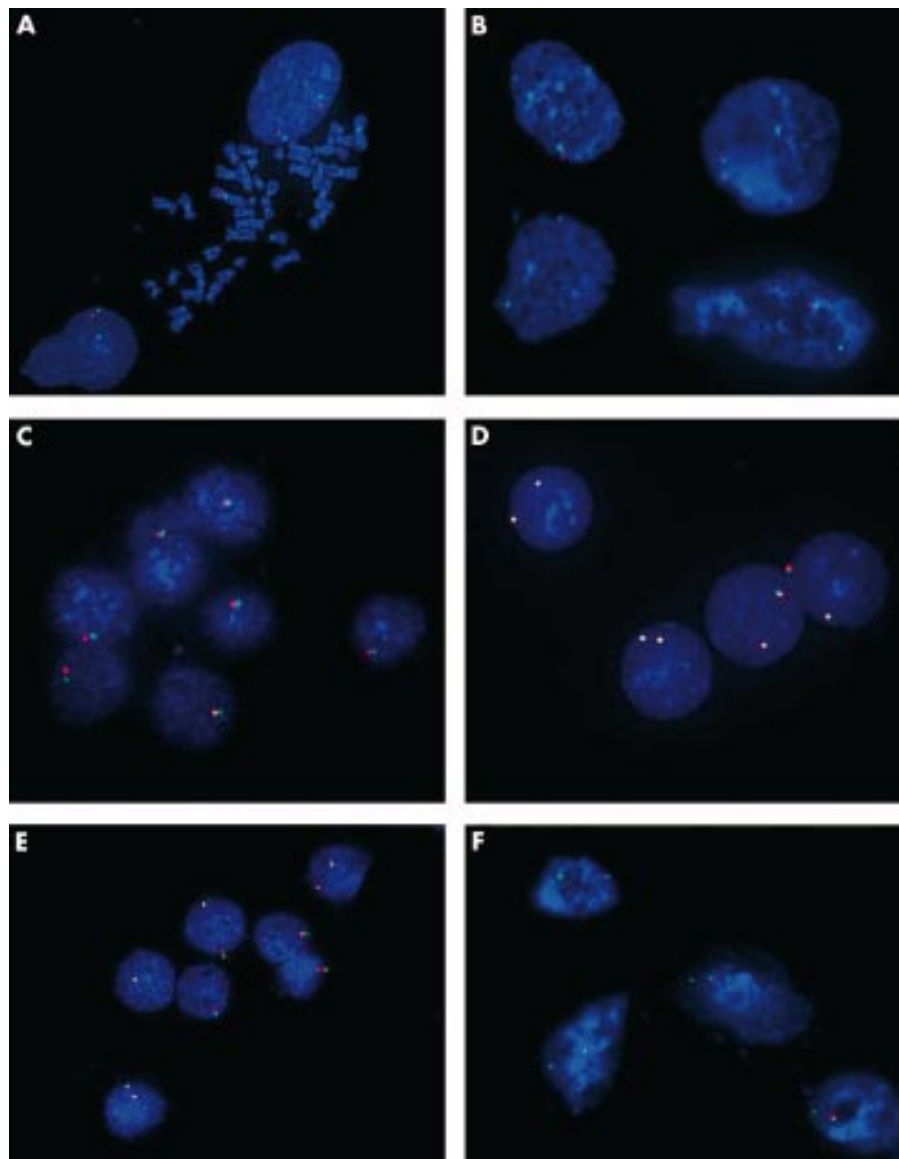


Figure 4 Deletion of 2p16 region in CNC tumours. (A) FISH with BAC 400-P-14 (red) and α -satellite probe specific for chromosome 2 (green) to fibromyxoma cells (case 12). A metaphase spread and a nucleus, located in the lower left of the image, showed deletion of only the 2p16 region; the centromeric α -satellite probe showed the expected two signals. A third cell was normal, showing two copies of each probe. (B) Interphase FISH to ovarian tumour cells (case 29) with BACs 400-P-14 (red) and 422-A-6 (green) showed deletion of BAC400-P-14; BAC 422-A-6 showed two signals. (C) FISH with BACs 400-P-14 (red) and 514-O-11 (green) to PPNAD cells (case 10) showed deletion of both BACs; these BACs were situated very close to each other on the physical map of 2p16 region. (D) Prostate adenoma cells used as control hybridised with BACs 400-P-14 and 514-O-11 showed two copies of both probes. (E) Ovarian tumour cells (case 41) hybridised with BACs 400-P-14 and 435-I-1 that had the partial overlap with BAC400-P-14 showed deletion of both BACs in four cells; the other three cells were normal, showing two copies of each probe. (F) Hybridisation of BACs 400-P-14 (red) and 406-J-5 (green) to PPNAD cells (case 10). Cells showed deletion of BAC 400-P-14 and two copies of 406-J-5.

specific for the centromere of chromosome 2. When possible, normal tissue associated with the CNC tumours was also hybridised with the corresponding BAC; no abnormalities were detected in these cells. Finally, control experiments included hybridisation of BAC clones from chromosomes 1, 4, 16, and 22 to CNC tumours. The expected two signals were detected in more than 90% of cells, suggesting that the observed 2p16 changes were probably specific for CNC tumours.

DISCUSSION

In this study, CGH showed a low level amplification of 2p as the most consistent abnormality in CNC tumours. One tumour showed amplification of 2p16-23 narrowing the region of search. Previous genetic mapping also narrowed the

affected region to an approximately 2 Mb long area between markers D2S2153 and D2S1352.⁷ Therefore, we examined the region for cytogenetic changes using FISH analysis of CNC tumours with the previously identified, as well as new, BACs from the 2p15-p21 contig.

Overall, more abnormalities were found in touch preparations than in primary cell lines, most likely the result of overgrowth of fibroblasts in cultures.³²⁻³⁴ Amplification of the region represented by HSRs occurred in 60% of the samples, deletions in 32%, and amplification and deletion of 2p16 in different cell populations from the same lesion in the remaining 8%. These data are consistent with the CGH data and our previous findings of inconsistent microsatellite alterations of the 2p16 region.³⁵ There have been no previous reports of consistently amplified or deleted chromosomal regions in CNC

tumours as determined by conventional cytogenetic analysis. Chromosome 2 was involved in some of the chromosome rearrangements that were seen in sporadic or associated with CNC myxomas.^{36,37} However, these changes were non-clonal and 2p16 was not specifically investigated.

Dual colour FISH with BACs from opposite sites of the *CNC2* region helped to establish the boundaries of the identified amplicon. Despite the extensive variability from tumour to tumour and even from cell to cell within the same tumour, amplification always included the region covered by BAC400-P-14, 435-I-1, and 514-O-11, although the latter was consistently less amplified than the former two. Interestingly, these BACs were also involved in all tumours that showed deletion of 2p16. Dual colour FISH using BAC 400-P-14 and other BACs in its proximity helped us to estimate the minimal size of the genomic region deleted in a number of CNC tumours. The region was covered by BAC 400-P-14 and overlapped with BAC435-I-1 and BAC514-O-11. Thus, the size of the deleted region was identical to that with the highest level of amplification.

To date, several ESTs from the 2p16 CNC critical region, including five ESTs that represent four different genes from BAC 400-P-14, have been identified and excluded as *CNC2* candidates⁹⁻¹¹ (Kirschner and Stratakis, unpublished data). The 2p21 region has been recently reported to harbour the protein kinase C-epsilon gene (*PKC-ε*) that is amplified in thyroid cancer.¹² We mapped BAC 1D9 containing this gene and found that it was located considerably more telomeric than *CNC2* and well above the region of chromosome 2 that was amplified or deleted in all CNC tumours (unpublished data).

The presence of amplification on 2p16-21 suggests the presence of an oncogene that may be responsible for tumorigenesis in CNC patients. The loss of this region in a number of CNC tumours would be similar to losses observed in other tumours caused by abnormal activation of an oncogene, such as *MET* in carcinomas,³⁸ and *RET* in pheochromocytomas.³⁹ We speculate that the oncogenic activity of this unidentified gene may be mediated by overexpression of the mutant allele in tumours where amplification is seen, or the exclusive expression of the mutant allele in the absence of the wild type allele in tumours where deletion is seen. Unfortunately, in the cases in which deletion was found, the disease was either sporadic or DNA was not available from affected relatives to test for the inheritance of the mutant versus that of the wild type allele.

Mechanisms that underlie the process of amplification are still not well known; it is generally believed to occur during the late stages of carcinogenesis. However, in our study, when amplification was detected it occurred mostly in benign tumours. This prompted us to look for a feature in the 2p16 region that would make it different from other genomic areas. In fact, 2p16 contains the relatively infrequently expressed, aphidicolin sensitive, fragile site *FRA2D*.⁸ Some models of amplification involve common fragile sites⁴⁰ which occur with varying frequencies in the population.⁴¹⁻⁴³ Such sites may predispose DNA to the genomic instability observed in cancer.⁴⁴ They may or may not affect the function of a gene. An example of this is that of the fragile histidine triad (FHIT), which maps to the *FRA3B* site on chromosome 3 and is involved in cell cycle regulation; its abnormalities have been associated with a variety of tumours.^{45,46} A different and less frequent fragile site on chromosome 11 has been associated with the common breakpoint identified in chronic lymphocytic leukaemia.⁴⁷ The difficulty we encountered in the genomic sequencing of 2p16 and its instability in tumours may be related to the presence of the *FRA2D* fragile site; characterisation of the latter will shed more light on its relevance to tumorigenesis.

The pattern of cytogenetic changes (amplification or deletion) on 2p16 does not seem to be associated with any clinical or histopathological features of the tumours studied. For example, among 20 PPNAD cases examined in this study, eight showed deletion, nine showed amplification, and two showed both. A remarkable observation is that the changes of the 2p16 region were observed in tumours from CNC patients, with and without *PRKARIA* mutations (mapping to 17q and 2p and elsewhere, respectively). We also analysed *CNC1* and *CNC2* tumours with regard to their type of cytogenetic abnormality (amplification or deletion); 18 of 23 tumours from a total of 28 *CNC1* tumours showed amplification and five showed deletion. From 14 of 16 *CNC2* tumours, an equal number of tumours (n=7) showed amplification or deletion. This is not a statistically significant difference (p=0.07) but it may represent a trend for 2p16 amplification to be more frequent in *CNC1* tumours.

Because the phenotypes of *CNC1* and *CNC2* patients are not significantly different, it has been suggested that both CNC genes could be involved in the same molecular pathway.⁴⁸ This study certainly supports this notion. From our results, the following mechanism of tumorigenesis in *CNC1* emerges (fig 5):

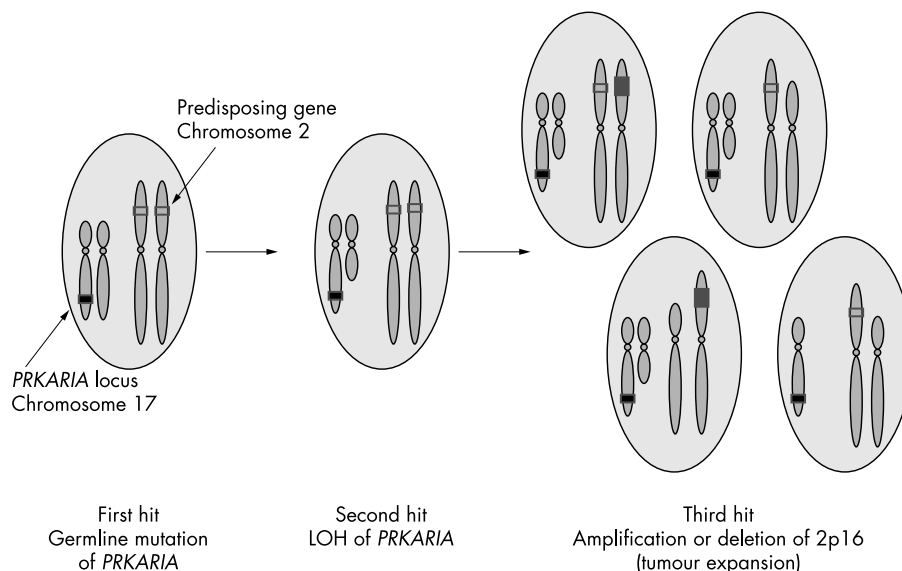


Figure 5 A diagram of potential molecular "hits" in *CNC1* tumours; details are provided in the Discussion (see text).

a germline mutation of *PRKARIA* serves as the "first hit", and LOH of *PRKARIA* as the second hit (although it may not always be necessary), whereas 2p alterations serve as yet another molecular event that may be necessary for tumour formation in this syndrome.

In summary, our results suggest the presence of putative candidate gene(s) on 2p16 that may be responsible for *CNC2* and have oncogenic function in at least a subset of families. Our data also imply that cytogenetic alterations in the 2p16 region may be one of the earliest events in the pathogenesis of *CNC* tumours.

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